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PRINCIPAL INVESTIGATOR: Dr. Jack Lichy

CONTRACTING ORGANIZATION: Armed Forces Institute of Pathology
Washington, DC 20306-6000

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Washington, DC 20306-6000

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This project aimed to determine at what stage of the progression of breast cancer loss of heterozygosity (LOH) is first observed and to determine whether LOH correlates with clinical behavior. Intraductal, infiltrating, and metastatic tumor components were isolated by microdissection from 115 breast cancers. Each microdissected specimen was analyzed for LOH at 14 loci known to show high frequency LOH in breast cancer. At each locus examined, LOH was usually seen in the intraductal component of the tumor and maintained throughout tumor progression. A significant association was found between LOH at D13S263 and positive lymph nodes. No correlations were observed between LOH at any of the markers and survival, ER status, tumor size, or tumor grade. In 21% of the cases, LOH results were not consistent with direct progression from intraductal to invasive to metastatic tumor, but instead provided evidence for divergent pathways of growth. Because LOH generally was seen early in tumor development, a group of non-malignant breast biopsies was studied for LOH. LOH was found to occur commonly in the components of fibrocystic disease, with the highest frequency observed in apocrine metaplasia. LOH was also observed in morphologically normal specimens, suggesting that LOH can occur during normal development.

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Jack W. Beaty 7/14/99

PI Signature Date

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INTRODUCTION

The earliest events in the pathogenesis of breast cancer typically involve the loss of a normal growth regulatory mechanism by a ductal or lobular epithelial cell. Progression of the disease through the stages of intraductal proliferation to invasive carcinoma and then to metastatic disease appears to require additional alterations in growth regulatory pathways. A substantial body of evidence now supports the idea that these alterations in growth regulation result from genetic events such as point mutation, deletion, and gene amplification [1-4]. Our study aims to characterize genetic alterations in breast tumors at the various stages of tumor progression. If metastasis requires additional genetic events beyond those responsible for the intraductal and invasive components of the tumor, one should find genetic alterations in the metastasis that are not present in primary tumor. Alternatively, there may be certain genetic lesions which occur early in tumor development that can predispose a tumor to metastasize without the acquisition of additional genetic defects. The identification of such a lesion would provide an important prognostic indicator, because it would provide a means for predicting the likelihood of the development of metastatic disease in tumors identified at an early stage. The characterization of genetic changes present in individual tumor components thus offers the possibility of identifying new prognostic indicators as well as helping to elucidate the significance of genetic events to tumor progression.

The type of genetic analysis performed in our study is the amplification of polymorphic loci by the polymerase chain reaction (PCR) [5]. This technique permits the detection of loss of heterozygosity (LOH) in tumor specimens relative to normal tissue from the same patient. LOH at specific loci has been observed frequently in breast cancer. High frequency of LOH for a specific genetic marker is thought to imply the presence of a tumor suppressor gene at that locus [3, 4]. In certain cases (e.g., p53 on 17p, DCC on 18q), the loss of one copy of the tumor suppressor gene (LOH) is found in association with mutation of the remaining copy. In such cases, LOH indicates that both copies of the tumor suppressor gene have become inactivated, resulting in the loss of a normal growth regulatory pathway. The PCR methodology also permits the detection of gene amplification, assuming that amplification involves only one of the two copies of the gene present. In breast cancer, amplification of the HER2/neu oncogene is of particular interest because of potential prognostic implications [2].

The general strategy of our study involves the identification of a group of breast cancer cases from the AFIP archives followed by microdissection of the intraductal, infiltrating, and metastatic components present in each tumor, and analysis of each tumor component for LOH at multiple genetic loci. The results should help address questions such as when during tumor progression specific genetic lesions occur, and whether LOH at any particular locus has value in predicting the course of progression of an individual tumor. In addition, through the analysis of multiple closely linked markers, the boundaries of each region of LOH can be identified. Comparison of multiple cases showing interstitial deletions often demonstrates a narrow region where these deletions overlap one another. The identification of such a region of overlap suggests the existence of a tumor suppressor gene in the common segment of overlapping LOH.

BODY

Experimental Methods. 115 cases diagnosed as carcinoma of the breast were retrieved from the AFIP archives. These cases were chosen from those submitted to the institute between 1975 and 1982 so that survival data could be generated over at least a 15 year time period from the initial diagnosis. Specimens were analyzed microscopically to identify regions of intraductal, infiltrating, and metastatic carcinoma, which were then isolated by microdissection. If available, a lymph node section was taken as the normal control for each case; otherwise, normal breast tissue was used. Tissue lysates containing PCR amplifiable DNA were prepared by a standard proteinase K digestion technique. This resulted in approximately four hundred and fifty specimens. These lysates were analyzed by PCR for the presence of polymorphic markers on chromosomes 3p, 9p, 11p, 13q, 16q, 17p, and 17q. The PCR primer sequences were obtained from the Genethon database. At least two markers were used for each of these loci. A more detailed study, aimed at narrowing the smallest region of overlap, was carried out for chromosome 11p15. For this study, the entire collection of lysates was analyzed for LOH at ten different polymorphic markers over an approximately 10 megabase region of 11p15. PCR products were labeled with ^{32}P by kinasing one of the primers. Reaction products were separated on a denaturing polyacrylamide gel and identified by autoradiography. A reduction in allele ratio of greater than 50% relative to the normal control was interpreted as loss of heterozygosity (LOH).

Results. As proposed in the Statement of Work, the microdissected specimens from this group of cases were tested for LOH at each of the loci in our panel. A detailed study of LOH at 11p15 has been completed and a manuscript describing the results has been published in the *American Journal of Pathology*. The data on 11p15 defined a smallest region of overlap between the markers D11S1318 and D11S4046, demonstrated that LOH at this locus usually occurs by the time the tumor has progressed to the stage of intraductal carcinoma, and argued that LOH at this locus has no correlation with the clinical behavior of the tumor.

The data on the other loci examined showed a similar pattern to that observed at 11p15, in that LOH was usually present at the intraductal carcinoma stage and maintained throughout subsequent stages of progression. We have not conducted detailed studies of these loci to characterize smallest regions of overlap as was done for 11p15 because such studies of each of these regions have appeared in the literature since we initiated our own work, and we felt that unless we devoted all our efforts to one locus we were unlikely to contribute anything novel by such studies. We have organized the data on LOH during progression for each locus examined into a summary table in preparation for publication. The table of results for 16q has been included as Appendix A as an example. In this table, cases are categorized by the most advanced tumor stage present. For each marker, results are first given as (# with LOH)/(# informative). The results are then divided by tumor stage, showing (# with LOH in specific tumor component)/(# with LOH in any component).

We have analyzed the results of our study for correlations with clinical parameters to determine whether LOH at any of these loci could be a useful prognostic indicator. More specifically, the data were analyzed for associations between LOH at each locus and (1) survival, (2) lymph node metastasis, (3) Estrogen receptor status, (4) tumor size, (5) tumor

grade, and (6) LOH at other loci. The observation of greatest potential importance was the significant correlation between LOH at the marker D13S263 with the presence of positive lymph nodes ($p=.004$). This marker was chosen because of its proximity to the RB1 gene, the gene responsible for familial retinoblastoma. Interestingly, LOH at the other chromosome 13 marker in our panel, D13S260, showed no significant correlation with lymph node status. D13S260 maps close to the BRCA2 gene. Although BRCA2 is mutated in a small fraction of hereditary breast cancers, it is very rarely involved in sporadic cancers such as those in our study. The RB1 gene, in contrast, is known to be mutated in a fraction of sporadic breast cancers. This fraction is estimated to be approximately 25%; more accurate estimates of the frequency have not been reported due to the difficulty in screening such a large gene for mutations. The observed association raises the possibility that mutation of the RB1 gene may be associated with the development of lymph node metastases. This observation seems worthy of further investigation.

Other significant associations observed included associations between 13q LOH and 16q LOH ($p<.001$); 17p LOH with 17q LOH ($p=.009$); and 16q LOH with 17q LOH ($p=.03$). No correlations were observed between LOH at any of the markers and survival, ER status, tumor size, or tumor grade.

In analyzing our LOH data, we noted several instances where LOH can be present in one tumor component but absent in a specimen representing a more advanced stage of tumor progression. This finding implies that the different tumor components present in surgical specimens do not necessarily represent subsequent stages in tumor progression, but rather divergent pathways of cellular proliferation. However, in each case where clonal divergence has been observed, our data are consistent with the possibility that both tumor components share a common precursor. The presence of genetically divergent clones in resected breast cancer specimens has been reported in two studies, one focusing on multiple foci of intraductal carcinoma [6], the other on asynchronous metastases [7]. By inferring the existence of a common precursor cell from shared genetic lesions in tumor components that have genetically diverged, it becomes possible to construct an "evolutionary tree" for each tumor analyzed. As a result of another recent study [8], which demonstrated that LOH can be observed in morphologically normal tissue adjacent to carcinoma, it seemed that such evolutionary trees could be extended back to include lesions earlier than intraductal carcinoma, such as benign proliferations and normal ducts and lobules.

Based on these considerations, we elected to extend our study by conducting additional microdissections of cases that had given evidence of genetic divergence, now including foci of normal and premalignant epithelium in addition to the malignant foci that were initially studied. We have carried out such extensive microdissections on six of these cases. The resulting lysates were characterized for LOH at a panel of markers which we knew worked well from our initial studies. The results from each case have been used to infer the degree of clonal relatedness of the different foci dissected from each tumor specimen. This analysis has revealed an unexpected degree of heterogeneity among tumor components presumed to represent successive stages of progression. Our data has also succeeded in reproducing the observation that LOH can be present in normal tissue adjacent to the carcinoma.

The data on genetic heterogeneity has been organized into a manuscript which has been

submitted for publication. Of the panel of 115 cases, 24 (21%) demonstrated genetically divergent clones during tumor progression. Clonal divergence was observed most commonly between intraductal and infiltrating tumor (17 cases), but was also demonstrated between infiltrating and metastatic tumor (11 cases). Divergent LOH patterns were observed with markers on one chromosomal arm in 16 cases, on two in 7, and on four in one, and was observed most commonly with markers on 17p, 17q, and 16q. Results from four of the cases subjected to more extensive microdissection are presented as evolutionary trees showing the probable course of accumulation of genetic abnormalities during progression.

In the final funding period of the grant, additional microdissections were performed to address the fundamental issue of this grant: When does LOH occur? Since LOH was sometimes observed at benign ductal epithelium adjacent to tumor, it was of interest to examine putative precursors of malignancy in cancer free breasts. Benign lumpectomy specimens provide a source for such lesions. Such specimens often contain the components of fibrocystic disease (cysts, apocrine metaplasia, adenosis) as well as proliferative lesions such as ductal hyperplasia and intraductal papilloma. Microdissected foci representing the components of fibrocystic disease from a panel of 35 benign lumpectomy specimens was examined for LOH at the fourteen chromosomal loci used previously to analyze tumor specimens. The specimens included 21 foci of ductal hyperplasia, one of atypical ductal hyperplasia, 20 of apocrine metaplasia, 23 of adenosis, and 3 of intraductal papilloma. In addition, benign ducts or lobules were available from each case. At each locus examined, LOH was observed with a frequency of 10-30%. There were two observations that were interesting and unexpected: (1) Benign TDLUs in these non-malignant breast biopsies demonstrated LOH at a frequency comparable to that observed in specimens of ductal hyperplasia and sclerosing adenosis; (2) The apocrine metaplasia specimens showed a significantly higher frequency of LOH than the other lesions examined. This high frequency of LOH in apocrine metaplasia has not been previously reported.

Based on the observation that apocrine metaplasia shows LOH with high frequency, we hypothesized that a focus of apocrine metaplasia adjacent to carcinoma could sometimes represent a genetic precursor. To test this hypothesis, slides from the panel of 115 cases of carcinoma of the breast which had been previously studied for LOH were reviewed to identify tumors with adjacent apocrine metaplasia. Fourteen such cases were identified. The foci of apocrine metaplasia were isolated by microdissection and analyzed for LOH at the same markers used to study the tumors. LOH was observed in 12 of the fourteen apocrine metaplasia specimens.

The results were interpreted in terms of their consistency with a precursor-product relationship and the strength with which the data supported such a relationship. In 10 of the 14 cases, the results were *consistent with* such a relationship in the sense that the tumor demonstrated LOH at all of the markers showing LOH in the apocrine metaplasia specimen, and may or may not have shown additional changes. The strongest evidence supportive of this relationship was provided by cases in which the tumor and apocrine metaplasia shared LOH at two or more loci but in which only the tumor showed LOH at additional loci. Three cases met these criteria. Also consistent with a precursor-product relationship were the results obtained with one case in which common LOH was detected at 11p but only the tumor showed LOH at 17p, as well as with four cases where the tumor and apocrine metaplasia specimen showed

identical LOH patterns.

In four other apocrine metaplasia specimens, the LOH results were not consistent with a simple precursor-product relationship. In one of these cases, the two specimens each demostrated LOH at one locus, but no common LOH was identified. In one case, common LOH was detected at three loci, but different alleles had been lost at an 11p marker, suggesting genetic divergence from a common precursor. In two cases, shared LOH was observed at multiple loci, but LOH at one locus in each case was observed only in the apocrine metaplasia specimen. The results in these two cases are consistent with genetic divergence from a common precursor or development of the apocrine metaplasia focus from the cells of the tumor. The results suggest that a focus of apocrine metaplasia can be a precursor in the development of cancer.

Conclusions. At the outset we hypothesized that certain genetic lesions would characteristically occur at specific stages of tumor progression. Our results argue that this hypothesis is false, that LOH at all of the loci examined occurs most commonly by the time the tumor has progressed to the intraductal carcinoma stage. Our data reveal no preferential order in which LOH occurs: LOH at any of the loci can occur early or late in progression. Analysis of the LOH data for clinical correlations revealed a significant correlation of potential importance between 13q LOH and positive lymph nodes. This finding raises the possibility of a role for RB1 or a closely linked gene in metastasis. With respect to LOH at 11p15, our data specifically refute the claims that this genetic lesion is a late event in breast cancer progression, and that it is a useful prognostic indicator, but confirm the localization of the smallest region of overlap reported by others. We have confirmed the observation that LOH can be detected in normal tissue adjacent to the carcinoma. By conducting extensive microdissections of cases showing genetic heterogeneity, we have shown that what we initially interpreted as lesions representing successive stages of progression present in surgically resected specimens often represent divergent pathways of tumor evolution. One important implication of this result is that it is now apparent that one can not assume that metastatic disease that develops years after resection of a primary tumor will contain the same genetic lesions present in the resected specimen. It will be important to take the genetic heterogeneity of breast cancer into consideration when developing strategies for early detection of recurrent disease that rely on detecting genetic alterations in the tumor. The analysis of biopsy specimens lacking cancer demonstrated that LOH is present in benign lobular epithelium even before morphologic changes are detectable. However, the frequency of LOH in benign lobules is much less than that in malignant tumors at all markers studied.

REFERENCES

1. Leslie, KO and Howard, P. 1992. Oncogenes and antioncogenes in human breast carcinoma. *Pathol. Ann.* 27 (Pt. 1):321-342.

2. Yokota, J, Yamamoto, T, Toyoshima, K, Terada, M, Sugimura, T, Battifora, H, and Cline, MJ. 1986. Amplification of c-*erbB-2* oncogene in human adenocarcinoma *in vivo*. *Lancet* 1:765-766.

3. Ali, IU, Lidereau, R, Theillet, C, and Callahan, R. 1987. Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. *Science.* 238:185-188.

4. Fearon, ER and Vogelstein, B. 1990. A genetic model for colorectal tumorigenesis. *Cell* 61:759-767.

5. Gruis, NA, Abel, ECA, Bardoel, AFJ, Devilee, P, Frants, RR, and Cornelisse, CJ. 1993. PCR-based microsatellite polymorphisms in the detection of loss of heterozygosity in fresh and archival tumour tissue. *Br. J. Cancer* 68:308-313.

6. Fujii, H, Marsh, C, Cairns, P, Sidransky, D, and Gabrielson, E. 1996. Genetic divergence in the clonal evolution of breast cancer. *Cancer Res.* 56:1493-1497.

7. Kuukasjarvi, T, Karhu, R, Tanner, M, Kahkonen, M, Schaffer, A, Nupponen, N, Pennanen, S, Kallioniemi, A, Kallioniemi, O, and Isola, J. 1997. Genetic heterogeneity and clonal evolution underlying development of asynchronous metastasis in human breast cancer. *Cancer Res.*57:1597-1604.

8. Deng, G, Lu, Y, Zlotnikov, G, Thor, AD, and Smith, HS. 1996. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. *Science.* 274:2057-2059.

BIBLIOGRAPHY

Publications:

1. Lichy, J.H., Zavar, M., Tsai, M.M., and Taubenberger, J.K. Loss of heterozygosity on chromosome 11p15 during histologic progression in microdissected ductal carcinoma of the breast. Am. J. Path. 153:271-278, 1998.

2. Lichy, J.H., Dalbegue, F., Zavar, M., Washington, C., Tsai, M.M., Sheng, Z, and Taubenberger, J.K. Genetic heterogeneity in ductal carcinoma of the breast. Submitted.

3. Washington, C., Dalbegue, F., Taubenberger, J.K., and Lichy, J.H. Loss of heterozygosity in microdissected components of fibrocystic disease: genetic evidence that apocrine metaplasia can sometimes be a precursor to carcinoma. In preparation.

4. Lichy, J.H., O'Leary, T.J., Dalbègue, F., Zavar, M., Sheng, Z., Tsai, M., and Taubenberger, J.K. Loss of heterozygosity during breast cancer progression: Analysis of microdissected tumor components. In preparation.

Abstracts:

1. Lichy, J.H., Dalbègue, F., Zavar, M., Tsai, M, Sheng, Z., and Taubenberger, J.K. "Genetic Heterogeneity in Ductal Carcinoma of the Breast." USCAP Meeting, San Francisco, March 1999.

2. Dalbègue, F., Zavar, M., Washington, C., Tsai, M., Sheng, Z.,and Taubenberger, J. K., and Lichy, J.H. Discordant patterns of LOH demonstrate the development of divergent clones during breast cancer progression. Poster presentation at the "Cancer Genetics and Tumor Suppressor Genes Conference," Frederick, MD, June 12-16, 1997.

3. Lichy, J. H., Dalbègue, F., Zavar, M., Washington, C., Tsai, M., Sheng, Z.,and Taubenberger, J. K. Characterization of Breast Cancer Progression by Analysis of Genetic Markers. Poster and platform presentation at conference: "Era of Hope: Department of Defense Breast Cancer Research Program." Washington, D.C., Oct. 30-Nov 4, 1997.

4. Lichy, J. H., Taubenberger, J.K., Zavar, M., Tsai. M. M. Optimal dinucleotide repeat markers for detecting loss of heterozygosity at chromosome 11p15 in breast cancer. Am. J. Pathol. 140:1786, 1996.

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Appendix A

Summary of LOH data during histologic progression for chr 16q

Histologic Diagnosis	No. Marker	No. Cases	LOH at 16q by stage of progression				
			Any Comp.	Intraductal	Invasive	Metastasic	Recurrent
Intraductal	D16S421	8	1/1(100%)	1/1(100%)	NA	NA	NA
	D16S496	8	1/3(34%)	1/1(100%)	NA	NA	NA
carcinoma only	D16S512	8	2/2(100%)	2/2(100%)	NA	NA	NA
Invasive carcinoma	D16S421	58	5/14(36%)	3/4(75%)	4/5(80%)	NA	NA
	D16S496	58	20/30(67%)	9/15(60%)	16/20(80%)	NA	NA
without metastases	D16S512	58	15/27(56%)	10/13(77%)	8/12(67%)	NA	NA
Invasive carcinoma	D16S421	43	11/15(73%)	7/8(88%)	8/11(73%)	7/11(64%)	NA
	D16S496	43	13/19(68%)	10/10(100%)	10/11(91%)	7/12(58%)	NA
with metastases	D16S512	43	14/26(54%)	8/11(73%)	9/13(69%)	10/14(71%)	NA
Invasive, metastases	D16S421	6	1/2(50%)	0/0	0/0	1/1(100%)	1/1(100%)
	D16S496	6	4/4(100%)	0/2	3/4(75%)	4/4(100%)	3/4(75%)
and recurrence	D16S512	6	0	0	0	0	0